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OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

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Cancer Assessment Review Committee
Health Effects Division (7509P)

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The Cancer Assessment Review Committee (CARC) met on February 13, 2013 to evaluate the cancer classification of tetraconazole in accordance with the *EPA's Final Guidelines for Carcinogen Risk Assessment* (March, 2005). Attached please find the final Cancer Assessment Document.

CANCER ASSESSMENT DOCUMENT

RE-EVALUATION OF THE CARCINOGENIC POTENTIAL OF ***TETRACONAZOLE***

PC Code 120603

FINAL

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

DATA PRESENTATION:

Anwar Dunbar, Toxicologist

DOCUMENT PREPARATION:

Kristin Rury, Executive Secretary

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Gregory Akerman

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Jess Rowland, Co-Chair

PV Shah

Charles Wood, Consulting Pathologist

Doug Wolf, Consulting Pathologist

Yintak Woo

NON-COMMITTEE MEMBERS IN ATTENDANCE:

Other Attendees: R. J. Bever, Carol Christensen, Jaime D'Agostino, Angela Howard, Minerva Mercado, Monique Perron

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EXECUTIVE SUMMARY

On February 13, 2013, the Cancer Assessment Review Committee (CARC) of the Health Effects Division (HED) of the Office of Pesticide Programs (OPP), met to re-evaluate the carcinogenic potential of Tetraconazole based on new data submitted to the Agency to support the registrant's proposed mode of action (MoA) for liver tumor formation in mice.

In July, 1999, the CARC classified tetraconazole as "likely to be carcinogenic to humans" based upon treatment related increases in liver tumors in both sexes in the mouse in the carcinogenicity study. No treatment-related tumors were observed in the rat carcinogenicity study. The CARC recommend the linear approach for quantification of cancer risk (TXR No. 0013948).

Dr. Anwar Dunbar of Risk Assessment Branch 1 (RAB1) presented the carcinogenicity data as well as recently submitted mode of action and other existing relevant mechanistic data to support the postulated constitutive androstane receptor (CAR)-mediated mitogenic mode of action (MoA) for liver tumors in mice.

Mode of Action (MoA)

The liver tumors observed in the mouse carcinogenicity study were observed in both males and females at 800 ppm and 1250 ppm (1250 pm was considered an excessive dose). The postulated MoA for hepatocellular tumorigenesis in tetraconazole-treated mice involves a nuclear receptor-mediated, non-genotoxic MoA. The key events in this MoA are activation of the constitutive androstane receptor (CAR, nuclear receptor), increased gene expression associated with an increase in microsomal enzyme activity, increased hepatocyte proliferation, and hepatocellular tumor formation. Activation of CAR and its translocation to the nucleus appears to be the initiating event in the MoA for tetraconazole.

To demonstrate the MoA, in a 28-day MoA study (MRID 48930401), tetraconazole was administered to groups of six male mice at dietary concentrations of 0, 90, 400, or 800 ppm for 1, 4, 14, or 28 days. Groups of 6 animals per dose group were sacrificed at the end of each treatment period and specific MoA parameters such as cell proliferation (BrdU labeling), cytochrome (CYP) P-450 expression and activity, liver weight and histopathology were evaluated. The dietary concentrations were equivalent to 0, 14.1-17.2, 57.6 -78.4, and 114.2-158.9 mg/kg bw/day, respectively.

Postulated Key Events for the MoA

The sequence of events in the proposed MoA for induction of liver tumors by tetraconazole is as follows:

- Activation of the constitutive androstane receptor (CAR) (**Key Event #1)**
- Increased expression of targeted genes **(Associated Event)**
- Induction of liver CYP enzymes **(Associated Event)**

- Increased liver weight and hepatocyte hypertrophy **(Associated Event)**
- Increased hepatocellular proliferation **(Key Event # 2)**
- Increased incidence of altered hepatocyte foci **(Key Event # 3)**
- Appearance of hepatocellular neoplasms **(Key Event # 4)**

The CARC concluded that there is sufficient evidence with dose and time concordance to support the postulated MoA for liver cell tumors in male and female mice.

Classification and Quantification of Carcinogenic Potential

The CARC considered the following factors in their weight-of-evidence deliberation on assessing the carcinogenic potential of Tetraconazole:

1. The liver tumors observed in male and female mice were considered to be treatment related;
2. There were no treatment-related tumors in male or female rat;
3. The hypothesized MoA (activation of the CAR receptor leading to cell proliferation) was supported by studies that clearly indentified the sequence of key events, dose-response concordance and temporal relationship to tumor type. There was convincing evidence that the hepatocellular carcinogenic effects are not likely to occur below a defined dose range. The MoA data met the criteria established by the Agency;
4. There is no mutagenicity concern based on the results from the *in vitro* and *in vivo* genetic toxicity studies; and
5. Data are insufficient to conclude that the proposed CAR-mediated MoA for rodent liver cell carcinogenesis is not relevant to humans.

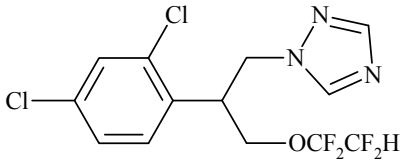
In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC classified tetraconazole as "Not likely to be carcinogenic to humans at levels that do not cause increased cell proliferation in the liver." Quantification of carcinogenic potential is not required. The current RfD of 0.0073 mg/kg/day is based on a 1 year dog study in which nephrotoxicity (organ weight and histopathology) were seen at 2.95 mg/kg/day (LOAEL) and no adverse effects were observed at 0.73 mg/kg/day (NOAEL). This RfD would be protective of any liver effects caused by tetraconazole in the mouse carcinogenicity or mode of action studies at higher doses.

I. INTRODUCTION

On February 13, 2013, the Cancer Assessment Review Committee (CARC) of the Office of Pesticide Programs met to re-evaluate the carcinogenic potential of tetraconazole. Tetraconazole was previously evaluated by the CARC in 1999 and was classified as "likely to be carcinogenic to humans". The registrant has subsequently submitted a 28-day mechanistic study (MRID No. 48930401) to support a proposed mode of action for liver tumors in mice, which is the subject of the 2013 CARC re-evaluation.

II. BACKGROUND

Tetraconazole is a systemic fungicide and is a member of the conazole/triazole class of pesticides. The PC Code is 120603 and the CAS Number is 112281-77-3. Tetraconazole acts by inhibiting the metabolic pathway leading to fungal sterol production (sterol-demethylation inhibitor (DMI)).



Summary of 1999 CARC Meeting (TXR No. 0013948)

On November 10, 1999, the CARC met to evaluate the carcinogenic potential of tetraconazole. The committee concluded the following:

• **Tetraconazole was not carcinogenic to rats.** The CARC determined that the thyroid tumors observed in male rats were not treatment-related because the increase in the tumor incidence (thyroid follicular cell adenomas) did not show statistical significance when compared with the concurrent controls, lacked a dose-response, and were within the range for the historical controls. There was no increase in thyroid or other tumors in females. The doses tested were determined to be adequate to assess the carcinogenic potential of tetraconazole in rats.

• **Tetraconazole was carcinogenic to mice** based on the following weight of evidence considerations: 1) There was a statistically significant ($p<0.01$) increase by pair-wise comparisons of the 800 ppm (118 and 140 mg/kg/day, for males and females, respectively) and 1250 ppm dose groups (217 and 224 mg/kg/day, for males and females, respectively) with the controls for hepatocellular adenomas, carcinomas (1250 ppm groups only) and combined adenomas/carcinomas in both sexes. The incidences of these tumors exceeded the range of historical controls. There were also statistically significant ($p<0.01$) increasing trends in both sexes for hepatocellular adenomas, carcinomas, and combined adenomas/carcinomas; 2) There was a numerical increase in the incidence of ovarian luteomas in high-dose females. A statistically significant ($p< 0.01$) increasing trend for luteomas was also evident. The incidence of luteomas (12%) was barely outside the historical control range (0%-8%). Nevertheless, the

incidence of 4 such tumors at the highest dose along with a positive trend was considered by some members to be biologically significant and supportive of carcinogenic potential of tetraconazole in mice. The dosing at 1250 ppm was considered by the Committee to be excessive due to increased mortality in both sexes. However, 800 ppm was considered to be an adequate dose since mortality was comparable to controls and the tumor incidences were significantly increased in both sexes. In addition, there was a decrease in body weight gain, increased liver and kidney weights and the presence of non-neoplastic changes in various organs including bone, liver, lungs, kidney, testes, epididymides and ovaries. No evidence was found for mutagenicity as a MoA for tetraconazole.

In accordance with the EPA *Draft Guidelines for Carcinogen Risk Assessment* (July, 1999), the Committee classified tetraconazole as **"likely to be carcinogenic to humans"** by the oral route based on the following weight-of-the-evidence considerations:

1. Increased incidences of liver tumors in male and female mice.

2. The relevance of the observed tumors to human exposure cannot be discounted.

3. Structurally related compounds are hepatocarcinogens in mice.

For human cancer risk assessment, the Committee recommended a linear low-dose extrapolation approach based on the combined hepatocellular tumors in male or female mice, whichever is more potent. This approach is supported by the lack of confirmation of the mode of action of tetraconazole.

III. MOUSE LIVER TUMORS PREVIOUSLY EVALUATED BY CARC

In a carcinogenicity study (MRID 44305305), M14360 (95.05% a.i.) was administered to 50 Crl:CD-1 (ICR) mice/sex/dose in their diet at dose levels of 0, 10, 90, 800, 1250 ppm (for males: 0, 1.4, 12, 118, 217 mg/kg/day; for females: 0, 1.6, 14.8, 140, 224 mg/kg/day) for 80 weeks. The CARC evaluated this carcinogenicity study previously (TXR#00013948).

A. Survival Analysis

Male and female mice had significant increasing trends for mortality with increasing doses of Tetraconazole. The statistical evaluation of mortality was based upon the Thomas, Breslow and Gart computer program (TXR#00013540).

Tetraconazole: Crl:CD-1 (ICR) Mouse Study
Male Mortality Rates and Cox or Generalized K/W Test Results.

	Weeks				
Dose (ppm)	1-26	27-34	35-52	53-81[f]	Total
0	1/50	0/49	1/49	10/48	12/50** (24)
10	0/50	0/50	3/50	14/47	17/50 (34)
90	1/50	0/49	2/49	8/47	11/50 (22)
800	1/50	0/49	1/49	9/48	11/50 (22)
1250	0/50	2/50	5/48	29/43	36/50** (72)

[+]Number of animals that died during interval/Number of animals alive at the beginning of the interval.

[f]Final sacrifice at week 81.

( )Percent.

Tetraconazole: Crl:CD-1(ICR) Mouse Study
Female Mortality Rates and Cox or Generalized K/W Test Results

	Weeks				
Dose (ppm)	1-26	27-34	35-52	53-83[f]	Total
0	1/50	0/49	0/49	8/49	9/50* (18)
10	0/50	0/50	0/50	11/50	11/50 (22)
90	0/50	0/50	0/50	9/50	9/50 (18)
800	1/50	0/49	0/49	7/49	8/50 (16)
1250	0/50	0/50	0/50	18/50	18/50* (36)

[+]Number of animals that died during interval/Number of animals alive at the beginning of the interval.

[f]Final sacrifice at week 81.

( )Percent.

Note: time intervals were selected for display purposes only. Significance of trend denoted at control. Significance of pair-wise comparison with control denoted dose level.
*If *, then p <0.05, if ** ; then p< 0.01.*

B. Discussion of Tumor Data

The statistical evaluation of mortality indicated significant increasing trends with increasing doses of tetraconazole in male and female mice. The results of tumor analyses for male and female mice are presented in Tables 1 and 2, respectively. Statistical evaluation of liver tumors in both sexes revealed a significant increasing trend with differences in the pair-wise comparisons of the 1250 ppm dose group with the controls for benign, malignant and benign and/or malignant tumors combined, all at $p < 0.01$. A statistically significant increased incidence of combined benign and malignant liver tumors was observed at 1250 ppm (86% for males and 65% for females) and 800 ppm (49% for males and 22% for females) compared to the control (20% for males and 0% for females). The tumor incidence in animals receiving ≤90 ppm was found to be similar to that of controls. In both sexes, there were also significant differences in the pair-wise comparisons of the 800 ppm dose group with the controls for liver benign tumors and for benign and/or malignant tumors combined, both at $p < 0.01$ (Tables 1 and 2). The increased incidence of liver tumors at both 800 and 1250 ppm was well outside the range for the historical controls (the incidence of hepatocellular adenomas in historical controls ranged from 7.7% - 24% in males and 0% - 1.9% in females and the incidence of hepatocellular carcinomas ranged from 0% - 14% in males and was 0% in females).

Female mice had a significant increasing trend in ovarian benign luteomas at $p < 0.01$, but no significant differences in the pair-wise comparisons of any dose group (Table 3). The incidence of ovarian tumors was 5 % and 12 % at 800 ppm and 1250 ppm, respectively. The incidence at 1250 ppm was slightly outside the historical control range (0% to 8 %). However, if the denominator of 50 instead of 32 was used, the incidence at high dose would be 8% (4/50) and within the historical control range. Nevertheless, the incidence of 4 such tumors at the highest dose along with a positive trend was considered by some committee members to be biologically significant and supportive of carcinogenic potential of tetraconazole in mice.

Table 1. Male Mice: Liver Tumor Rates+ and Peto's Prevalence Test Results.					
ppm	**0**	**10**	**90**	**800**	**1250**
mg/kg/day	**0**	**1.4**	**12**	**118**	**217**
Benign	9/49	8/50	6/49	22/49	34[a]/49
%	(18)	(16)	(12)	(45)	(69)
p =	0.000**	-	-	0.003**	0.000**
Malignant	1/48	2/47	2/47	4/48	20[b]/45
%	(2)	(4)	(4)	(8)	(44)
p =	0.000**	0.398	0.059	0.134	0.000**
Combined	10/49	9[c]/50	7[c]/33	24[d]/49	42[e]/49
%	(20)	(18)	(14)	(49)	(86)
p =	0.000**	-	-	0.002**	0.000**

[+]Number of tumor-bearing animals/Number of animals examined, excluding those that died or were sacrificed before observation of the first tumor.
[a]First liver benign tumor observed at Week 34, 1250 ppm.
[b]First liver malignant tumor observed at Week 50, dose 1250 ppm.
[c]One animal in each of the 10 and 90 ppm dose groups had both an adenoma and a carcinoma.
[d]Two animals in the 800 ppm dose group had both an adenoma and a carcinoma.
[e]Twelve animals in the 1250 ppm dose group had both an adenoma and a carcinoma.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 2. Female Mice: Liver Tumor Rates[+] and Peto's Prevalence Test Results.					
ppm	**0**	**10**	**90**	**800**	**1250**
mg/kg/day	**0**	**1.6**	**14.8**	**140**	**224**
Benign	0/49	0/49	0/50	11/49	26[a]/49
%	(0)	(0)	(0)	(22)	(53)
p =	0.000**	-	-	0.000**	0.000**
Malignant	0/48	0/47	0/50	1/49	17[b]/44
%	(0)	(0)	(0)	(2)	(39)
p =	0.000**	-	-	0.162	0.000**
Combined	0/49	0/49	0/50	11[c]/49	32[d]/49
%	(0)	(0)	(0)	(22)	(65)
p =	0.000**	-	-	0.000**	0.000**

[+]Number of tumor-bearing animals/Number of animals examined, excluding those that died or were sacrificed before observation of the first tumor.
[a]First liver benign tumor observed at Week 57, 1250 ppm.
[b]First liver malignant tumor observed at Week 64, dose 1250 ppm.
[c]One animal in the 800 ppm dose group had both an adenoma and a carcinoma.
[d]Eleven animals in the 1250 ppm dose group had both an adenoma and a carcinoma.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 3. Female Mice: Ovarian Tumor (Luteomas) Rates[+] and Peto's Prevalence Test Results.					
ppm	**0**	**10**	**90**	**800**	**1250**
mg/kg/day	**0**	**1.6**	**14.8**	**140**	**224**
Benign	2/41	0/39	0/41	2/42	4/32
%	(5)	(0)	(0)	(5)	(12)
p =	0.006**	-	-	-	0.121

[+]Number of tumor-bearing animals/Number of animals examined, excluding those that died or were sacrificed before observation of the first tumor.
First ovarian benign luteomas observed at Week 81, in final sacrifice animals, concurrently at all doses.

IV. 2013 CARC's EVALUATION OF THE PROPOSED MODE OF ACTION (MoA) FOR INDUCTION OF HEPATOCELLULAR TUMORS IN MICE

The liver tumors observed in the mouse carcinogenicity study were observed in both males and females at 800 ppm (1250 pm was considered an excessive dose). The postulated mode-of-action (MoA) for hepatocellular tumorigenesis in tetraconazole-treated mice involves a nuclear receptor-mediated, non-genotoxic MoA. The key events in this MoA involve activation of the constitutive androstane receptor (CAR, nuclear receptor), increased gene expression associated with an increase in microsomal enzyme activity, increased hepatocyte proliferation, and hepatocellular tumor formation. Activation of the CAR and its translocation to the nucleus appears to be the initiating event in the MoA for tetraconazole.

A. Postulated Key Events for the MoA

The sequence of events in the proposed MoA for induction of liver tumors by tetraconazole is as follows:

- Activation of the constitutive androstane receptor (CAR) (**Key Event #1)**
- Increased expression of targeted genes **(Associated Event)**
- Induction of liver CYP enzymes **(Associated Event)**
- Increased liver weight and hepatocyte hypertrophy **(Associated Event)**
- Increased hepatocellular proliferation **(Key Event # 2)**
- Increased incidence of altered hepatocyte foci **(Key Event # 3)**
- Appearance of hepatocellular neoplasms **(Key Event # 4)**

B. Studies Available for the MoA Analysis

Several studies that describe the effect of tetraconazole on the liver are available for establishing the postulated MoA. These studies are briefly described below.

Previous studies considered in the 1999 CARC decision:

1. Carcinogenicity study in Crl:CD-1 (ICR) mice (MRID 44305305): Tetraconazole was administered for 80 weeks to male mice at dietary concentrations of 0, 10, 90, 800, or 1250 ppm (0, 1.4, 12, 118, and 217 mg/kg bw/day, respectively), to female mice at 0, 1.6, 14.8, 140 ppm (224 mg/kg bw/day, respectively). Parameters examined included mortality, clinical signs, body weight, weight gain, feed consumption, hematology parameters, gross pathologic lesion, non-neoplastic and neoplastic histopathologic lesions, and organ weights including the liver.

2. Subchronic toxicity study in Crl:CD-1 (ICR)BR mice (MRID 44778701): Tetraconazole was administered for 13 weeks to male and female mice at dietary concentrations of 0, 5, 25, 125, or 625 ppm (0, 1, 4, 16, and 85 mg/kg bw/day for males and 0, 1, 4, 20, or 103 mg/kg bw/day for females). Parameters examined included mortality, clinical signs, body weight, weight gain, feed consumption, limited serum chemistry parameters, gross pathological lesions, liver, brain, testes, and kidney weight, and histopathology of the liver and kidneys.

3. Liver enzyme induction in Crl:CD-1 (ICR)BR mice (MRID 44751309): Tetraconazole was administered for 4 weeks to male and female mice at dietary concentrations of 0, 20, 800, or 1250 ppm (0, 3.9, 150, and 225 mg/kg bw/day for males and 0, 4.6, 175, and 293 mg/kg bw/day for females). Phenobarbital (75 mg/kg bw/day) was the positive control. Endpoints evaluated included mortality, clinical signs, body weight, weight gain, feed consumption, gross pathological lesions, liver weight, and liver microsomal cytochrome P450 enzyme activities.

Recently submitted study to support the proposed mode of action for liver tumors:

4. Mode-of-action study in Crl:CD-1 (ICR) mice (MRID 48930401): The MoA for liver tumor formation was investigated with regards to dose- and time-response relationships. Tetraconazole was administered in the diet of male mice (only) at dietary concentrations of 0, 90, 400, or 800 ppm (0, 15.2, 69 and 132.6 mg/kg/day) for 1, 4, 14, or 28 days. Weight normalized doses for tetraconazole and phenobarbital are shown in Table 4. Parameters examined included mortality, clinical signs, body weight, weight gain, feed consumption, liver weight, gross and microscopic liver lesions, liver cell proliferation, CAR localization, limited clinical chemistry, focused gene expression, and microsomal enzyme activities.

Table 4. Dose of Tetraconazole to Male Mice (mg/kg bw/day).

Conc. in diet (ppm)	Duration of treatment				
	1 Day	4 Days	14 Days	28 Days	Average Dose*
Tetraconazole					
0	0	0	0	0	0
90	17.2	14.4	15.0	14.1	15.2
400	65.3	75.2	78.4	57.6	69
800	114.2	138.1	158.9	119.0	132.6
Phenobarbital					
1000	154.5	168.7	172.3	152.7	169.1

[a] Data taken from pages 17 and 27 of the study report (MRID 48930401).
*Calculated by the health effects division.

C. Data Supporting the Key Events in the MoA for Tetraconazole

1. Key Event # 1: Activation of the constitutive androstane receptor (CAR)

In male mice administered tetraconazole for 4 days, CAR translocation into the nuclei of hepatocytes was demonstrated by an immunohistochemical staining technique using rabbit polyclonal anti-CAR antibody (MRID 48930401). No evidence of CAR translocation was seen mice at 90 ppm. The first evidence of CAR nuclear translocation occurred at 400 ppm in 1/6 mice administered tetraconazole. Definitive evidence of CAR activation and translocation into the nucleus of hepatocytes was found in 4/6 mice receiving tetraconazole at 800 ppm. The effect of tetraconazole on CAR translocation was similar to that of PB at 1000 ppm. Nuclear staining occurred predominately in the centrilobular hepatocytes of tetraconazole- and PB-treated mice. Data showing translocation of CAR to hepatocyte nuclei are presented in Table 5.

Table 5. Translocation of the constitutive androstane receptor to liver cell nuclei of male mice.

Score [a]	Tetraconazole				PB
	0	90 ppm (15.2 mg/kg/day)	400 ppm (69 mg/kg/day)	800 ppm (132.6 mg/kg/day)	1000 ppm (162.1 mg/kg/day)
4-Day Treatment					
1	6	6	5	2	1
2	0	0	1	0	3
3	0	0	0	3	2
4	0	0	0	1	0
Average score [b]	1.00	1.00	1.17	2.50	2.17

Data obtained from page 39 in the study report (MRID 48930401).
[a] Score (positive stained cells): 1 = 0-5 cells; 2 = 6-10 cells; 3 = 11-20 cells; and 4 = 21-50 cells.
[b] The average score calculated by the reviewer based on the number of nuclei stained.

2. Associated Event: Expression of Targeted Genes

The CAR translocation to hepatocyte nuclei results in increased expression of targeted genes (*Cyp2b10* and *Cyp3a11*). Male mice were administered tetraconazole for 1, 4, 14, or 28 days. TaqMan quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the levels of mRNA associated with specific genes (*Cyp2b10*, *Cyp3a11*, and *Ugt2b38*) (MRID 48930401). The expression of *Cyp2b10* showed a dose-related increase after treatment with tetraconazole. *Cyp2b10* expression was increased by about 5- to 14-fold in mice treated with 90 ppm, but the increases were statistically significant only after treatment with tetraconazole for 1 and 14 days (about 9- and 14-fold, respectively) compared with that of controls. *Cyp2b10* was also significantly increased after treatment with tetraconazole at 400 and 800 ppm for 1, 4, 14, and 28 days compared with that of a vehicle control. The level of increase was about 22- to 27-fold at 400 ppm and about 31- to 44-fold at 800 ppm compared with that of a vehicle control. A similar increase in the expression of *Cyp2b10* was observed in mice treated with PB. The expression of *Cyp3a11* also showed a dose-related increase compared with the control. No significant increase was observed at 90 ppm, but the expression of *Cyp3a11* was significantly increased throughout the study in the groups fed the 400-ppm (about threefold) and 800-ppm diets (about fourfold).

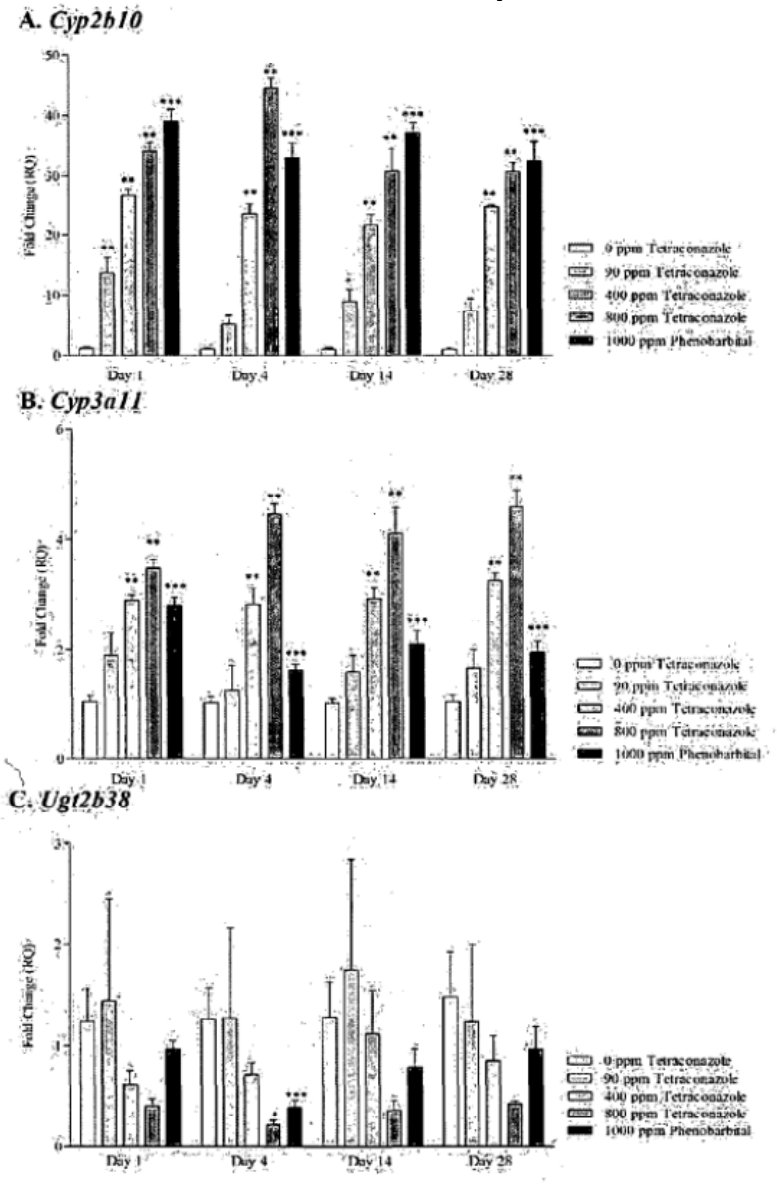
Likewise, PB also increased the expression of *Cyp3a11* throughout the study but the level was slightly below that seen in tetraconazole-treated mice. In contrast, the expression of *Ugt2b38* was inhibited by exposure to tetraconazole and PB compared with that of controls. The inhibition of *Ugt2b38* showed a negative dose-related trend that reached statistical significance only on day 4 in the group fed the 800-ppm tetraconazole or PB diet. These results show that tetraconazole and PB have similar effects on the expression of *Cyp2b10*, C*yp3a11*, and *Ugt2b38*. The data showing effects of tetraconazole on gene expression are presented in Table 6 and Figure 1.

Table 6. Gene expression (relative mRNA levels) in hepatic cells assessed by qRT-PCR [a].

Duration of Treatment	Tetraconazole				PB
	0	90 ppm (15.2 mg/kg/day)	400 ppm (69 mg/kg/day)	800 ppm (132.6 mg/kg/day)	1000 ppm (162.1 mg/kg/day)
No. of animals/group	**6**	**6**	**6**	**6**	**6**
Cyp2b10					
1-Day Treatment	1.17 [a]	13.7**	26.6**	34.2**	39.2*
4-Day Treatment	1.10	5.15	23.5**	44.4**	33.1*
14-Day Treatment	1.12	9.01*	21.7**	30.8**	37.1*
28-Day Treatment	1.03	7.39	24.7**	30.8**	32.5*
Cyp3a11					
1-Day Treatment	1.04	1.90	2.89**	3.48**	2.80*
4-Day Treatment	1.03	1.25	2.82**	4.47**	1.63*
14-Day Treatment	1.02	1.60	2.93**	4.12**	2.10*
28-Day Treatment	1.04	1.67	3.25**	4.60**	1.97*
Ugt2b38					
1-Day Treatment	1.24	1.44	0.611	0.402	0.972
4-Day Treatment	1.26	1.27	0.715	0.216*	0.394*
14-Day Treatment	1.28	1.75	1.12	0.360	0.785
28-Day Treatment	1.48	1.23	0.848	0.425	0.972

Data taken from Figure 6 (p.42) and Appendix XI (pp. 146-149) in the study report (MRID 48930401).
[a] Fold increase (mean rounded to three significant figures) compared with vehicle control group (not otherwise explained by the investigator) that was equal to 1.000 for each gene.
*p<0.05 (Dunnett's or t-test), **p<0.01 (Dunnett's test), statistically significant compared with controls.

Figure 1. Relative Levels of mRNA for Specific Genes Determined by qRT-PCR



Bars represent mean fold increase ± SD

*Means are statistically different compared to the basal diet control ($p < 0.05$, Dunnett's test)

**Means are statistically different compared to the basal diet control ($p < 0.0001$, Dunnett's test)

***Means are statistically different compared to the basal diet control ($p < 0.05$, t test)

3. Associated Event: Induction of Hepatic Microsomal Enzyme Activities

The activity of microsomal enzymes associated with the targeted gene expression have been investigated in mice administered tetraconazole for 1 to 28 days. PB was the positive control to show similarity in the MoA.

Male mice were administered tetraconazole at dietary concentrations of 0, 90, 400, or 800 ppm for 1, 4, 14, or 28 days to assess the effect on microsomal enzymes in the liver, which included that 7-benzoxyresorufin-O-debenzylase (BROD, CYP3A isoenzyme), 7-pentoxyresorufin-O-dealkylase (PROD, CYP2B isoenzyme), and 7-Ethoxyresorufin-O-deethylase (EROD, CYP1A1 isoenzyme). PB administered in the diet at 1000 ppm was the positive control. BROD enzyme activity was significantly increased in mice fed the 90-ppm diet for 4 and 28 days, the 400-ppm diet for 1, 4 and 28 day, the 800-ppm diet for 14 and 28 days compared with that of controls. PROD enzyme activity was significantly increased in mice fed the 90- or 400-ppm diets for 4 and 28 days and in mice fed the 800-ppm diet for 28 days compared with that of controls. EROD enzyme activity was not significantly affected in mice fed any concentration of tetraconazole compared with that of controls. BROD and PROD activities were significantly increased in PB-treated mice after 1, 4, 14, and 28 days, and the activity of EROD activity was significantly increased after 1, 4, and 28 days compared with that of controls (MRID 48930401). The results of this study are presented in Table 7 and Figure 2.

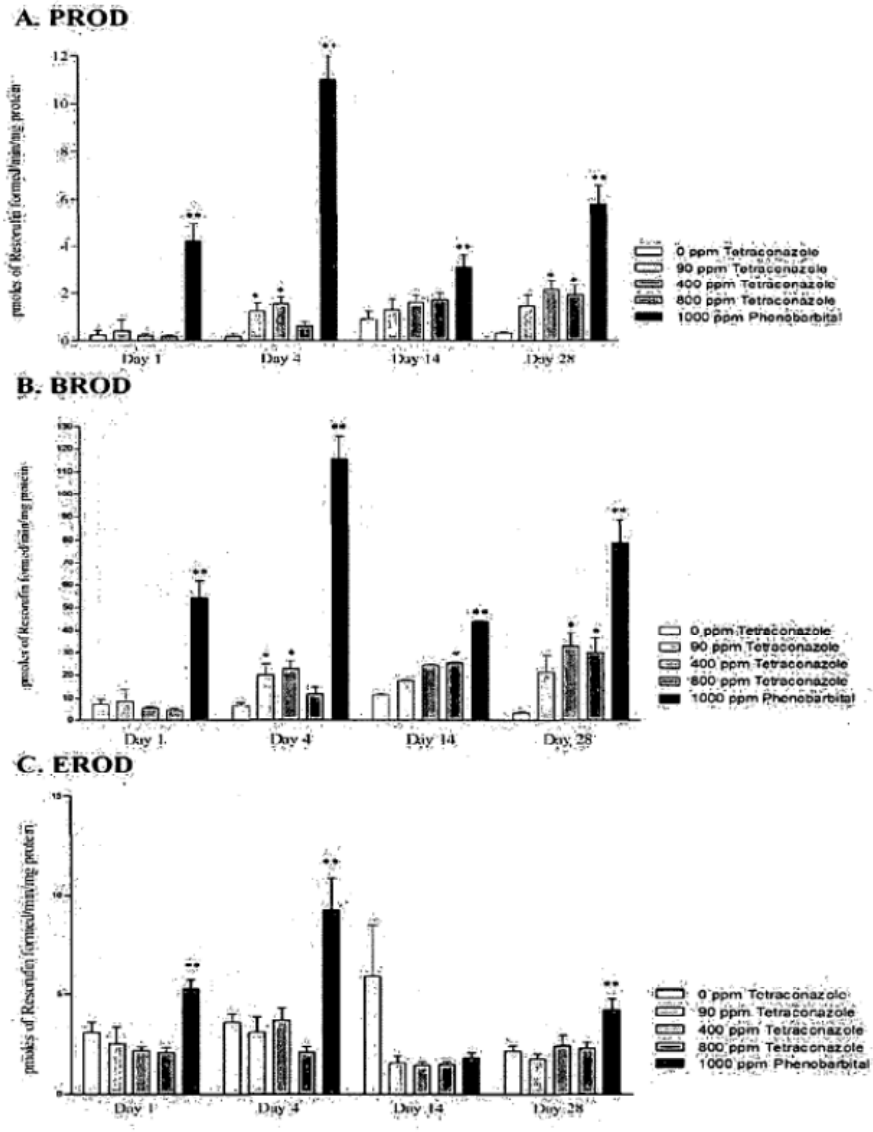
Table 7. Liver microsomal enzyme activity in mice treated with tetraconazole or phenobarbital for up to 28 days [a].

Duration of treatment	Tetraconazole, ppm (mg/kg/day)				PB ppm (mg/kg/day)
	0 (basal diet)	90 ppm (15.2 mg/kg/day)	400 ppm (69 mg/kg/day)	800 ppm (132.6 mg/kg/day)	1000 ppm (162.1 mg/kg/day)
No. animals/group	6	6	6	6	6
BROD [b]					
1-Day Treatment	7.359 ± 6.046	8.597 ± 5.349	5.444 ± 1.461	4.533 ± 1.481	54.280 ± 18.790*
4-Day Treatment	6.601 ± 3.750	20.037 ± 4.968*	22.711 ± 8.725*	11.773 ± 7.512	115.651 ± 24.650*
14-Day Treatment	11.429 ± 11.469	17.837 ± 7.579	24.565 ± 11.284†	25.493 ± 5.428*	43.935 ± 18.535*
28-Day Treatment	3.347 ± 0.963	21.404 ± 7.215††	32.768 ± 14.604*	30.069 ± 15.442*	79.016 ± 24.043*
PROD [b]					
1-Day Treatment	0.244 ± 0.498	0.427 ± 0.467	0.239 ± 0.168	0.192 ± 0.172	4.223 ± 1.750*
4-Day Treatment	0.203 ± 0.303	1.253 ± 0.322*	1.553 ± 0.714*	0.615 ± 0.454	11.003 ± 2.426*
14-Day Treatment	0.915 ± 0.818	1.303 ± 0.468	1.624 ± 0.746	1.744 ± 0.632	3.147 ± 1.218*
28-Day Treatment	0.341 ± 0.074	1.477 ± 0.460†	2.176 ± 0.973*	1.945 ± 0.942*	5.812 ± 1.902*
EROD [b]					

Table 7. Liver microsomal enzyme activity in mice treated with tetraconazole or phenobarbital for up to 28 days [a].					
Duration of treatment	**Tetraconazole, ppm (mg/kg/day)**				**PB ppm (mg/kg/day)**
	0 (basal diet)	**90 ppm (15.2 mg/kg/day)**	**400 ppm (69 mg/kg/day)**	**800 ppm (132.6 mg/kg/day)**	**1000 ppm (162.1 mg/kg/day)**
No. animals/group	**6**	**6**	**6**	**6**	**6**
1-Day Treatment	3.116 ± 1.266	2.595 ± 0.797	2.241 ± 0.491	2.128 ± 0.658	5.228 ± 1.369*
4-Day Treatment	3.628 ± 0.998	3.136 ± 0.742	3.720 ± 1.456	2.153 ± 0.686	9.296 ± 3.863*
14-Day Treatment	5.964 ± 6.257	1.571 ± 0.384	1.444 ± 0.434	1.487 ± 0.325	1.867 ± 0.636
28-Day Treatment	2.221 ± 0.606	1.760 ± 0.308	2.472 ± 1.288	2.348 ± 0.763	4.221 ± 1.405*

[a] Data taken from Figure 7 (p. 44) and Appendix XII (pp. 151-162) in the study report (MRID 48930401).
[b] Enzyme activity expressed as pmoles/min/mg protein (mean ± SD).
*p<0.05, statistically significant compared with controls.
†p<0.05, statistically significant compared with controls, calculated by the reviewer (Dunnett's or t-test)

Figure 2. Enzyme Activity



Bars represent mean ± SD
*Means are statistically different compared to the basal diet control ($p<0.05$, Dunnett's test)
**Means are statistically different compared to the basal diet control ($p<0.05$, t test)

In another 4-week study (MRID 44751309), liver microsomal enzyme activities were assessed in male and female mice administered tetraconazole in the diet at concentrations of 0, 20, 800, or 1250 ppm. PROD activity in mice administered tetraconazole was significantly increased at all doses in male and female mice, whereas ethylmorphine N-demethylase (EMND) and p-nitrophenyl UDP-glucuronyltransferase (p-nitrophenyl UDPGT) activities were significantly increased only at 800 and 1250 ppm. The increases in enzyme activities were dose related up to 800 ppm, but did not increase or showed a decrease relative to the lower doses at 1250 ppm. PB

produced a similar response on PROD activity as seen with tetraconazole. These results are summarized in Table 8.

Table 8. Microsomal enzyme activity in the liver of mice administered tetraconazole in the diet [a].

Enzyme Activity Measured	Tetraconazole ppm (mg/kg bw/day), [M/F]			PB
	20 ppm (3.9/ 4.6)	800 ppm (150/ 175)	1250 ppm (225/ 293)	75 mg/kg bw/day
28-Day Treatment				
Males				
7-Pentoxyresorufin-O-dependentylase (PROD)				
nmoles/min/mg protein	5.0**	7.7**	6.0**	70.3
nmoles/min/g liver	4.9**	9.1**	6.5**	88.8
7-Ethoxyresorufin-O-deethylase (EROD)				
nmoles/min/mg protein	0.9	0.9	1.1	2.7
nmoles/min/g liver	1.0	1.2	1.3*	3.7
Ethylmorphine N-demethylase (EMND)				
nmoles/min/mg protein	1.2	2.3**	2.3**	4.4
nmoles/min/g liver	1.3	3.0**	2.7**	6.0
p-Nitrophenyl UDPGT				
nmoles/min/mg protein	1.0	1.2**	1.2**	1.0
nmoles/min/g liver	1.1	1.6**	1.4**	1.4
Females				
7-Pentoxyresorufin-O-dependentylase (PROD)				
nmoles/min/mg protein	2.0*	2.3*	1.8*	22.9
nmoles/min/g liver	2.2**	3.2**	2.7**	32.4
7-Ethoxyresorufin-O-deethylase (EROD)				
nmoles/min/mg protein	1.1	1.3*	1.3*	5.3
nmoles/min/g liver	1.2	1.8*	2.0**	7.5
Ethylmorphine N-demethylase (EMND)				
nmoles/min/mg protein	1.5**	3.1**	2.6**	6.9
nmoles/min/g liver	1.7*	4.4**	4.0**	9.9
p-Nitrophenyl UDPGT				
nmoles/min/mg protein	1.1	1.4**	1.2**	1.2
nmoles/min/g liver	1.2*	1.9**	1.8**	1.7

[a] Data taken from pages 96 and 97 of the study report (MRID 44751309)

4. Associated Events: Increased Liver Weight and Hepatic Hypertrophy

Liver weight was measured and hepatocellular hypertrophy was assessed in male mice administered tetraconazole at 0, 90, 400, or 800 ppm for 1, 4, 14, and 28 days. Male mice treated similarly with 1000 ppm PB served as the positive control. Liver weight was not significantly affected in mice administered tetraconazole at 90 ppm. Liver weights were significantly increased throughout the study in mice treated with 400 ppm (+15% to +36%) compared with that of controls. At 800 ppm, liver weights were also significantly increased from day 4 to 28 of the study (+56% to +68%). Liver weight was significantly increased (+15% to +53%) throughout the study in PB-treated mice (MRID 48930401). Liver weights in mice treated with tetraconazole for 1 to 28 days are presented in Table 9.

Table 9. Liver weights (g ± SD) in male mice treated with tetraconazole or phenobarbital [a].					
Duration of Treatment	**Tetraconazole ppm (mg/kg bw/day)**				**PB (mg/kg bw/day)**
	0 (basal diet)	**90 ppm (15.2)**	**400 ppm (69)**	**800 ppm (132.6)**	**1000 ppm (162.1)**
No. animals/group	**6**	**6**	**6**	**6**	**6**
1-Day Treatment	1.85 ± 0.16	1.96 ± 0.12	2.12 ± 0.17* (+15) [b]	2.09 ± 0.25 (+13)	2.12 ± 0.19* (+15)
4-Day Treatment	1.90 ± 0.09	2.00 ± 0.13	2.59 ± 0.13* (+36)	3.10 ± 0.20* (+63)	2.87 ± 0.24* (+51)
14-Day Treatment	2.01 ± 0.23	2.07 ± 0.11	2.62 ± 0.40* (+30)	3.37 ± 0.22* (+68)	2.75 ± 0.34*(+37)
28-Day Treatment	1.98 ± 0.17	2.14 ± 0.13	2.56 ± 0.16* (+29)	3.09 ± 0.25* (+56)	3.02 ± 0.31* (+53)

[a] Data obtained from page 29 in the study report (MRID 48930401).
[b] Numbers in parentheses are the percent difference from control values.
*p<0.05, statistically significant compared with controls.

Centrilobular hypertrophy was not observed in male mice administered tetraconazole at 90 ppm for any duration up to 28 days. At 400 ppm, minimal centrilobular hypertrophy was observed in one mouse treated with tetraconazole for 1 day and all six mice treated with tetraconazole for 4 days; minimal to mild centrilobular hypertrophy was observed in all mice treated with the 400-ppm diet for 14 and 28 days. At 800 ppm, all mice had minimal centrilobular hypertrophy after treatment for 1 day and mild to moderate centrilobular hypertrophy after treatment for 4 to 28 days. The increase in average severity was dose related. There was also a time-dependent increase in severity of centrilobular hypertrophy in mice administered tetraconazole up to 14 days. All mice treated with PB had minimal to moderate centrilobular hypertrophy after treatment for 4 and 14 days; the lesion progressed to severe in all animals treated for 28 days. These results show that tetraconazole and PB have similar effects on centrilobular hypertrophy in mice (MRID 48930401). The incidence and severity of hepatic centrilobular hypertrophy are presented in Table 10.

Table 10. Incidence and average severity of hepatic centrilobular hypertrophy in male mice treated with tetraconazole or phenobarbital [a]					
Duration of Treatment	**Tetraconazole, ppm (mg/kg bw/day)**				**PB ppm (mg/kg bw/day)**
	0 (basal diet)	**90 ppm (15.2)**	**400 ppm (69)**	**800 ppm (132.6)**	**1000 ppm (162.1)**
No. animals/group	**6**	**6**	**6**	**6**	**6**
1-Day Treatment	0/6	0/6	1/6 (1.00)[b]	6/6 (1.00)	1/6 (1.00)
4-Day Treatment	0/6	0/6	6/6 (1.00)	6/6 (2.50)	6/6 (1.90)
14-Day Treatment	0/6	0/6	6/6 (1.83)	6/6 (2.83)	6/6 (2.50)
28-Day Treatment	0/6	0/6	6/6 (1.50)	6/6 (2.67)	6/6 (4.00)

[a] Data obtained from page 34 in the study report (MRID 48930401).
[b] Average severity calculated by the reviewer (1 = minimal, 2 = mild, 3 = moderate, 4 = severe)

In a 28-day study in male and female mice administered tetraconazole in the diet at concentrations of 20, 800, or 1250 ppm, relative liver weight was significantly increased at 800 and 1250 ppm but not at 20 ppm. Relative liver weight was not significantly affected in mice treated with PB at 75

mg/kg bw/day for 28 days. The effect of tetraconazole on liver weight in mice is presented in Table 11.

Table 11. Relative liver weight in mice administered tetraconazole or PB for 28 days [a].			
Tetraconazole [b] (mg/kg bw/day), [M/F]			PB
20 ppm (3.9/ 4.6)	800 ppm (150/ 175)	1250 ppm (225/ 293)	75 mg/kg bw/day
28-Day Treatment			
Male mice			
1.0	2.1**	2.7**	1.3
Female mice			
1.1	2.2**	2.6**	1.3

[a] Data obtained from MRID 44751309.
[b] Dietary concentrations in the mouse study.
**p<0.01, statistically significant compared with controls.

In a subchronic study, male and female mice administered tetraconazole in the diet at 0, 5, 25, 125, or 625 ppm for 90 days had significantly increased adjusted liver weights. The absolute, adjusted, and relative (to BW) weights were markedly increased (+66% to +77%) in both sexes at 625 ppm and slightly increased (13% to 15%) in females at 125 ppm. No effect was observed at 5 or 25 ppm. Gross pathological examination revealed enlarged livers in all males and females at 625 ppm. Microscopic examination showed minimal to moderate centrilobular hepatocellular hypertrophy in both sexes at 25, 125, and 625 ppm (MRID 44778701). The results of this study are presented in Table 12.

Table 12. Liver weight, incidence of liver enlargement, and incidence and average severity of centrilobular hepatocellular hypertrophy in male and female mice treated with tetraconazole [a].					
Parameter	Tetraconazole, (mg/kg bw/day, [M/F])				
	0 ppm (0)	5 ppm (1/1)	25 ppm (4/4)	125 ppm (16/20)	625 ppm (85/103)
90-Day Treatment					
Males					
Liver weight Absolute (g) Adjusted (g) Relative (% of BW)	1.81 1.83 5.25	1.68 1.62 4.65	1.81 1.86 5.34	1.97 1.93 5.52	3.21 (+77) [b] 3.22** (+77) 9.20 (+75)
Gross Examination Liver enlargement [c]	0	0	0	0	10
Histopathology Centrilobular hepatocellular [c]	0	0	7 (1.0) [d]	9 (1.4)	10 (3.0)
Females					
Liver weight Absolute (g) Adjusted (g) Relative (% of BW)	1.57 1.57 5.65	1.61 1.62 5.85	1.66 1.65 5.95	1.78 (+13) 1.80** (+15) 6.47 (+15)	2.65 (+69%) [b] 2.63**(+68%) 9.40 (+66)

Table 12. Liver weight, incidence of liver enlargement, and incidence and average severity of centrilobular hepatocellular hypertrophy in male and female mice treated with tetraconazole [a].					
Parameter	**Tetraconazole, (mg/kg bw/day, [M/F])**				
	0 ppm (0)	**5 ppm (1/1)**	**25 ppm (4/4)**	**125 ppm (16/20)**	**625 ppm (85/103)**
Gross Examination Liver enlargement [c]	0	0	0	0	10
Histopathology Centrilobular hepatocellular [c]	0	0	6 (1.3) [d]	9 (1.0)	10 (2.4)

[a] Data obtained from Tables, 7, 8, and 9 (MRID 44778701).
[b] Numbers in parentheses are percent difference from control values.
[c] Number of animals affected.
[d] Mean severity
N = 10

In an 80-week mouse study, tetraconazole was administered in the diet at concentrations of 0, 10, 90, 800, or 1250 ppm. Absolute liver weights showed a dose-related increase in both sexes. At 90 ppm, absolute liver weight was slightly but significantly increased in both sexes, whereas absolute liver weight was markedly increased at 800 and 1250 ppm in both sexes. Relative liver weight also showed a dose-related increase in both sexes administered tetraconazole for 80 weeks. Centrilobular hepatocyte enlargement (hypertrophy) was observed in a large number of male mice at 90 and 800 ppm (18/50 and 22/50, respectively), but was observed in only 2/50 males at 1250 ppm. Generalized hepatocyte hypertrophy was observed in 26/50 and 47/50 males, respectively, at 800 and 1250 ppm. In female mice, centrilobular hepatocyte hypertrophy was observed in only 8/50 animals at 800 ppm, 1/50 at 1250 ppm and none of the animals in the remaining dose groups. Generalized hepatocyte hypertrophy was observed in 19/50 and 44/50 female mice at 800 and 1250 ppm, respectively. Granulomatous inflammation, indicative of a toxic lesion was observed in both sexes at 800 and 1250 ppm (MRID 44305305). These data are presented in Table 13.

Table 13. Liver weight, incidence of liver enlargement, and incidence and average severity of centrilobular hepatocellular hypertrophy in male and female mice treated with tetraconazole for 80 weeks [a].					
Parameter	**Tetraconazole, (mg/kg bw/day, [M/F])**				
	0 ppm	**10 ppm (1.4/1.6)**	**90 ppm (12/14.8)**	**800 ppm (118/140)**	**1250 ppm (217/224)**
Males					
Liver weight (g)	2.14	2.15	2.39** (+12%)	5.21** (+143%)	10.00** (+367%)
Liver weight/body weight ratio	0.05	0.05	0.06	0.14	0.26
Histopathology Centrilobular hepatocyte enlarg. [c] Generalized hepatocyte enlarg.	 4 3	 5 1	 18** 7	 22** 26**	 2** 47**
Granulomatous inflammation	7	3	6	28*	33*
Females					
Liver weight (g)	1.70	1.88	1.97* (+16%)	3.74** (+120%)	6.24** (267%)
Liver weight/body weight ratio	0.05	0.05	0.06	0.11	0.20

Table 13. Liver weight, incidence of liver enlargement, and incidence and average severity of centrilobular hepatocellular hypertrophy in male and female mice treated with tetraconazole for 80 weeks [a].					
Parameter	**Tetraconazole, (mg/kg bw/day, [M/F])**				
	0 ppm	**10 ppm (1.4/1.6)**	**90 ppm (12/14.8)**	**800 ppm (118/140)**	**1250 ppm (217/224)**
Histopathology					
Centrilobular hepatocyte enlarg. [c]	0	0	0	8**	1
Generalized hepatocyte enlarg.	1	0	0	19**	44**
Granulomatous inflammation	2	3	4	15**	24**

[a] Data obtained from Table 4 (MRID 44305305).
[b] Numbers in parentheses are percent different from the control values.
[c] Number of animals affected.
**p<0.01, statistically significant compared with the control group.
N = 50

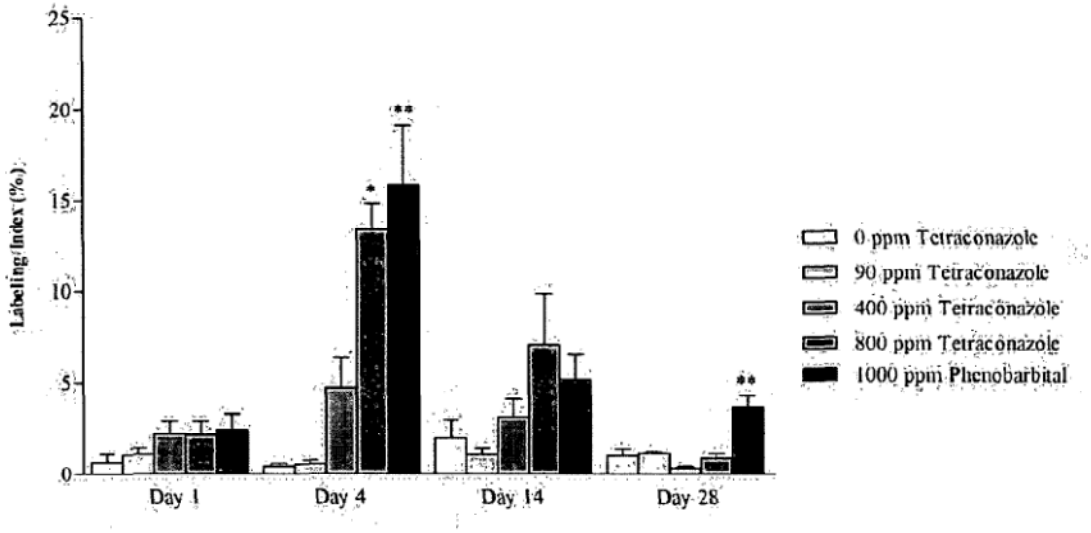
5. Key Event # 2: Increased Hepatocellular Proliferation

Hepatocellular proliferation was investigated in male mice administered tetraconazole in the diet at 0, 90, 400, or 800 ppm for 1, 4, 14, or 28 days. Hepatocellular proliferation was measured by BrdU incorporation into hepatic cell nuclei (hepatic labeling index). Treatment with tetraconazole at 90 or 400 ppm had no statistically significant effect on hepatic labeling indices at any duration of treatment. The only statistically significant increase in the hepatic labeling index was in mice administered tetraconazole at 800 ppm for 4 days. The hepatic labeling index appears to have peaked after treatment with the 400- and 800-ppm diet for 4 days and showed a dramatic decrease with continued treatment for 28 days. Treatment with PB also caused a statistically significant increase in the hepatic labeling index after treatment for 4 and 28 days; a decrease in the labeling index was also observed with continued treatment with PB for 14 and 28 days. There was considerable variability in these data as evidenced by the large standard deviations (MRID 48930401). These results are presented in Table 14 and Figure 3.

Table 14. Hepatic labeling index (cell proliferation) in male mice treated with tetraconazole or phenobarbital [a].					
Treatment day	**Tetraconazole (mg/kg bw/day)**				**PB (mg/kg bw/day)**
	0 (basal diet)	**90 ppm (15.2)**	**400 ppm (69)**	**800 ppm (132.6)**	**1000 ppm (162.1)**
No. animals/group	**6**	**6**	**6**	**6**	**6**
Day 1	0.64 ± 1.06 [b]	1.06 ± 0.87	2.18 ± 1.75	2.11 ± 1.92	2.41 ± 2.10
Day 4	0.39 ± 0.38	0.54 ± 0.54	4.70 ± 3.93	13.34 ± 3.61*	15.87 ± 7.93*
Day 14	1.95 ± 2.41	1.03 ± 0.85	3.11 ± 2.38	7.06 ± 6.80	5.14 ± 3.32
Day 28	0.97 ± 0.94	1.11 ± 0.20	0.31 ± 0.22	0.87 ± 0.59	3.66 ± 1.50*

[a] Data obtained from Table 12 (p. 35) in the study report (MRID 48930401).
*p<0.05, statistically significant (Dunnett's or t-test) from controls.

Figure 3. Hepatic Labeling Index



Bars represent mean ± SD
*p<0.05 compared to basal diet control (Dunnett's test)
**p<0.05 compared to basel diet control (t test)

Figure taken from page 36 of the study report (MRID 48930402).

6. Key Event # 3: Increased incidence of Altered Hepatic Foci

In male mice treated with tetraconazole ranging from 5- 625 ppm for 90 days, no basophilic, eosinophilic, or clear cell foci were observed during microscopic examination of the liver. Three of ten female mice treated with 625 ppm had moderate mid-zonal vacuolation (data not shown). Treatment of mice with tetraconazole at 800 or 1250 ppm for 80 weeks resulted in increased incidences of basophilic and eosinophilic (with and without vacuolization) hepatocytes in both sexes. The results are summarized in Table 15.

Table 15. Hepatocellular foci in the liver of CD-1 mice treated with tetraconazole for 80 weeks [a].

Parameter	Tetraconazole [b]				
	0 ppm	10 ppm	90 ppm	800 ppm	1250 ppm
Male Mice: 80-Week Treatment					
Basophilic hepatocytes	5	4	5	15*	17**
Eosinophilic hepatocytes ± vacuolation [c]	2	3	7	29**	18**
Female Mice: 80-Week Treatment					
Basophilic hepatocytes	1	0	0	9**	13**

Table 15. Hepatocellular foci in the liver of CD-1 mice treated with tetraconazole for 80 weeks [a].					
Parameter	**Tetraconazole [b]**				
	0 ppm	**10 ppm**	**90 ppm**	**800 ppm**	**1250 ppm**
Eosinophilic hepatocytes ± vacuolation [c]	0	0	0	12**	29**

[a] Data obtained from MRID 44305305.
[b] Dietary concentrations for mice
[c] Number of animals affected.
*p<0.05, **p<0.01, statistically significant compared with the control group.
N = 10 for 90-day treatment; N = 50 for 80-week treatment.

7. Key Event # 4: Hepatocellular Neoplasms

The incidences of benign and benign/malignant hepatocellular tumors combined were significantly increased in CD-1 male mice treated with tetraconazole at 800 and 1250, and the incidence of malignant tumors alone was significantly increased only as 1250 ppm. The first benign liver tumor in male mice was found at week 34 and the first malignant tumor was found at week 50 in the 1250-ppm group. In CD-1 female mice, the incidences of benign and benign/malignant hepatocellular tumors combined were also significantly increased in the 800- and 1250-ppm groups, and the incidence of malignant tumors was significantly increased only in the 1250-ppm group. The first benign tumor in females was found at week 57 and the first malignant tumor was found at week 64 in the 1250-ppm group. The trend test showed statistically significant trends for benign, malignant, and combined tumors in both sexes treated with tetraconazole (MRID 44305305). A summary of the hepatocellular tumor incidences is found in Table 16.

Table 16. Incidence of hepatocellular tumors in mice treated with tetraconazole [a].					
	Tetraconazole				
	0 (basal diet)	**10 ppm**	**90 ppm**	**800 ppm**	**1250 ppm**
Males					
Benign	9/49** (18%)[b]	8/50 (16%)	6/49 (12%)	22/49** (45%)	34/49** (69%)
Malignant	1/48** (2%)	2/47 (4%)	2/47 (4%)	4/48 (8%)	20/45** (44%)
Combined	10/49** (20%)	9/50 (18%)	7/33 (33%)	24/49** (49%)	42/49** (86%)
Females					
Benign	0/49**	0/49	0/50	11/49** (22%)	26/49** (53%)
Malignant	0/48**	0/47	0/50	1/49 (2%)	17/44** (39%)
Combined	0/49**	0/49	0/50	11/49** (22%)	32/49** (65%)

[a] Data obtained from (MRID 44305305 and HED DOC. No. 013948).
[b] Number of tumor-bearing animals/No. animals examined excluding those that died or sacrificed before first tumor found.; numbers in parentheses are incidence expressed as percentage.
*p<0.05, *p<0.01, statistically significant compared with the control group; the statistical significance for the trend test is in the control column.

8. Other Effects of Tetraconazole in Mice

In male CD-1 mice administered tetraconazole at dietary concentrations of 0, 90, 400, or 800 ppm for 1, 4, 14, or 28 days, serum alanine aminotransferase (ALT) activity was increased by more than 200% after treatment with 800 ppm for 4 days. No increase in ALT activity was noted with continued treatment for 14 or 28 days. In addition, sorbital dehydrogenase activity was elevated by more than 100% after treatment with 800 ppm for 4 days and by 83% after treatment with 800 ppm for 28 days. Increased ALT and SDH activities are indicators of liver toxicity. Microscopic evidence of liver toxicity was not reported in male mice administered tetraconazole in the diet (90-800 ppm) for up to 28 days (MRID 48930401).

In a 90-day subchronic study in mice administered tetraconazole in the diet, serum ALT activity was elevated two-fold in males at 625 ppm and by more than two- and fourfold in females at 125 and 625 ppm, respectively. Microscopic evidence of liver toxicity in the 90-day study included single cell necrosis, single cell degeneration, and necrosis in males and females and hepatocyte vacuolation in females at 625 ppm, and single cell degeneration in males and single cell necrosis in females at 125 (MRID 44778701).

In the 80-week carcinogenicity study in mice, microscopic evidence of liver toxicity included granulomatous inflammation, hepatocyte vacuolation, fat deposition, and bile duct hyperplasia at 800 and 1250 ppm. Serum enzymes were not evaluated in the carcinogenicity study (MRID 44305305).

D. Dose-Response and Temporal Association for Key Events and Tumorigenesis

The dose-response and temporal association for key events involved in the development of liver tumors are presented in Tables 17 and 18. Key events 1 and 2 and associated events, CAR translocation to hepatocyte nuclei, increased gene expression and corresponding enzyme activity, increased liver weight and centrilobular hypertrophy, and cell proliferation, occurred at or below doses and prior to development of liver tumors. The shortest latency for formation of hepatocellular adenomas was 34 weeks for male mice and 57 weeks for female mice. Key events 1, 2, and 3 were observed prior to week 34.

Table 17. Temporal and dose response for MoA key events related to male and female mouse liver tumors										
Dose Concordance ↓ ↓ ↓ ↓	**Temporal Concordance →→→→**									
	Dose (ppm)	**Key Event 1**					**Key Event 2**		**Key Event 3**	**Key Event 4**
		CAR Nuclear Translocation, *Cyp2b10* & *Cyp3A11* Transcripts & Enzyme Activity		**Associated Increased Liver Weight & Centrilobular Hypertrophy**			**Increased Hepatocyte Proliferation**		**Increased Incidence of Altered Foci**	**Hepatocellular Neoplasms**
		1-4 Days	**14-28 Days**	**1-4 Days**	**14-28 Days**	**90 Days - 2 Yrs**	**1-4 Days**	**14-28 Days**	**90 Days - 80 weeks**	**80 weeks**
	5-10	n/a	n/a	n/a	--	--	n/a	n/a	--	--
	20-25	n/a	**X**	n/a	--	**X**	n/a	n/a	n/a	n/a
	60-90	**X**	**X**	--	--	**X**	--	--	--	--
	125	n/a	n/a	n/a	n/a	**X**	n/a	n/a	n/a	n/a
	360-400	**X**	**X**	**X**	**X**	--	--	--	n/a	n/a
	625-640	n/a	n/a	n/a	--	**X**	n/a	n/a	n/a	n/a
	800*	**X**	**X**	**X**	**X**	**X**	**X**	--	**X**	**X**
	≥1250	n/a	**X**	n/a	**X**	**X**	n/a	n/a	**X**	**X**
	X = indicate a statistically significant effect compared with concurrent controls. -- indicate no statistically significant effect at the doses tested. * Indicate dose where statistically significant increase in tumor response; n/a = not applicable, doses not tested									

Table 18. Temporal sequence of key events in mice treated with tetraconazole										
Key Event	**Parameter**	**Tetraconazole (ppm)**								
		5-10	**20-25**	**60-90**	**125**	**360-400**	**625-640**	**800**	**≥1250**	**PB**
1 Day										
1	CAR translocation									
	↑ *Cyp2b10* expression			X	--	X	--	X	--	X
	↑ *Cyp3a11* expression			0	--	X	--	X	--	X
	↑ BROD activity			0	--	0	--	0	--	X
	↑ PROD activity			0	--	0	--	0	--	X
	↑ Liver weight			0	--	X	--	0	--	X
	Centrilob. hypertrophy			0	--	X	--	X	--	X
2	↑ Hepatocellular prolif.			0		0		0		
3	Hepatocellular foci									
4	Hepatocellular tumors									
4 Days										
1	CAR translocation			0	--	X	--	X	--	X
	↑ *Cyp2b10* expression			0	--	X	--	X	--	X
	↑ *Cyp3a11* expression			0	--	X	--	X	--	X
	↑ BROD activity			X	--	X	--	0	--	X
	↑ PROD activity			X	--	X	--	0	--	X
	↑ Liver weight			0	--	X	--	X	--	X
	Centrilob. hypertrophy			0	--	X	--	X	--	X

Table 18. Temporal sequence of key events in mice treated with tetraconazole										
Key Event	**Parameter**	**Tetraconazole (ppm)**								
		5-10	**20-25**	**60-90**	**125**	**360-400**	**625-640**	**800**	**≥1250**	**PB**
2	↑ Hepatocellular prolif.			0	--	0	--	X	--	--
2	Hepatocellular foci									
4	Hepatocellular tumors									
14 Days										
1	CAR translocation									
	↑ *Cyp2b10* expression			X	--	X	--	X	--	X
	↑ *Cyp3a11* expression			0	--	X	--	X	--	X
	↑ BROD activity			0	--	X	--	X	--	X
	↑ PROD activity			0	--	0	--	0	--	X
	↑ Liver weight			0	--	X	--	X	--	X
	Centrilob. hypertrophy			0	--	X	--	X	--	X
2	↑ Hepatocellular prolif.			0		0		0		
3	Hepatocellular foci									
4	Hepatocellular tumors									
28 Days										
1	CAR translocation									
	↑ *Cyp2b10* expression			0	--	X	--	X	--	X
	↑ *Cyp3a11* expression			0	--	X	--	X	--	X
	↑ BROD activity			X	--	X	--	X	--	X
	↑ PROD activity		X	--	--	X	--	X	X	X
	↑ EROD activity			--	--	--	--	X	X	
	↑ Liver weight					X		X	X	X
	Centrilob. hypertrophy			0	--	X	--	X	X	X
	↑ Hepatocellular prolif.			0	--	X	--	X	--	--
3	Hepatocellular foci			0		0		0		
4	Hepatocellular tumors									
	CAR translocation									
90 Days										
1	CAR translocation									
	↑ *Cyp2b10* expression									
	↑ *Cyp3a11* expression									
	↑ BROD activity									
	↑ PROD activity									
	↑ Liver weight		--	--	X	--	X	--	--	
	Centrilob. hypertrophy		X	--	X	--	X	--	--	
2	↑ Hepatocellular prolif.									
3	Hepatocellular foci									
4	Hepatocellular tumors									
80 Weeks - 2 Years										
1	CAR translocation									
	↑ *Cyp2b10* expression									
	↑ *Cyp3a11* expression									
	↑ BROD activity									
	↑ PROD activity									
	↑ Liver weight	0	--	X	--	--	X	X	X	

Table 18. Temporal sequence of key events in mice treated with tetraconazole										
Key Event	Parameter	Tetraconazole (ppm)								
		5-10	20-25	60-90	125	360-400	625-640	800	≥1250	PB
	Centrilob. hypertrophy	0	--	X	--	--	--	X	X	
2	↑ Hepatocellular prolif.									
3	Hepatocellular foci	--	--	--	--	--	--	X	--	
4	Hepatocellular tumors	0	--	0	--	--	--	X	X	

X = effect in mouse species; Xr = effect in rat species; 0 = tested, but no effect; -- = not tested at this dose; shaded area = not tested at any dose or no effect at all doses tested for the specified duration.

E. Strength, Consistency and Specificity of Tumor Response with Key Events

The data supporting the key events for hepatocellular tumorigenesis in tetraconazole-treated mice are consistent with the proposed MoA. The translocation of CAR to hepatocyte nuclei occurred early during treatment (as early as 4 days) and was dose related. The translocation of CAR was accompanied or followed by increases in *Cyp2b10* and *Cyp3a11* gene expressions, increased microsomal PROD and BROD activities, increased liver weight, and increased incidence severity of centrilobular hypertrophy, and increased hepatic cell proliferation. These results are consistent with the CAR activation as the molecular initiating event in the MoA. These events were similar to those observed when mice were treated with PB. In addition, the studies showed that the parameters occurring in Key Events #1 and #2 preceded the first observation of a hepatocellular tumor in mice at 34 weeks. Only male mice were used in the MoA study (MRID 48730401) submitted by the sponsor; male mice had more robust tumor response and appeared to be slightly more sensitive than females in the mouse carcinogenicity study (MRID 44305305). Therefore, the use of only males in the MoA study should not affect the strength of the data.

F. Biological Plausibility and Coherence

The postulated MoA for tetraconazole-induction of hepatocellular tumors in mice is coherent and plausible. The postulated MoA is a mitogenic MoA, which has been shown to induce hepatocellular tumors via non-genotoxic MoA involving CAR activation. Since the effects observed prior to the appearance of hepatocellular tumor in mice treated tetraconazole are similar to those observed in PB-treated mice, this MoA is considered plausible. The effects accompanying and following CAR translocation into hepatocyte nuclei precedes the appearance of tumors.

G. Alternative Modes-of-Action for liver tumors

Mutagenicity: No evidence was found for mutagenicity as a MoA for tetraconazole. Tetraconazole was negative in the following genetic toxicity tests: (1) Reverse gene mutation test in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 with and without S9 activation (MRID 44335511); (2) *In vitro* mammalian gene mutation assay in L5178Y mouse lymphoma cells with and without S9 activation (MRID 44335508); (3) *In vitro* chromosome aberration assay in Chinese hamster ovary (CHO) cells with and without S9 activation (MRID 44335507); (4) *In vitro* unscheduled DNA synthesis assay in cultured HeLa

cells with and without S9 activation (MRID 44335510); and (5) *In vivo* mouse micronucleus assay in male and female CD-1 mice administered single oral doses (185-740 mg/kg bw) of tetraconazole by gavage (MRID 44335509).

Other Receptor-Mediated MoAs: There are other receptor mediated pathways which could be involved in Liver tumorigenesis as well including the Aryl Hydrocarbon Receptor (AhR), the Pregnane X Receptor (PXR), and the Peroxisome Proliferation Activation Receptor Alpha (PPARα). Determination of activation of these receptors would require additional work on the part of the registrant. No evidence of increased peroxisomes (suggestive of a PPARα MoA) was reported.

Cytotoxicity/Regenerative Cell Proliferation: The studies in mice showed that serum enzyme activity indicative of liver toxicity (SDH) was elevated after treatment with tetraconazole for 28 days but was not accompanied by microscopic evidence of toxicity or increased proliferation. After treatment of mice with tetraconazole for 90 days, serum enzyme activities were elevated and microscopic evidence of liver toxicity was observed. The 80-week carcinogenicity study also revealed microscopic evidence of liver toxicity along with centrilobular hypertrophy and hepatocellular tumorigenesis. However, no evidence of regenerative proliferation was reported in any of these studies. Cytotoxicity/regenerative cell proliferation does not appear to be an alternative MoA for tetraconazole.

H. Uncertainties, Inconsistencies, and Database Limitations

One potential uncertainty in the MoA for tetraconazole is the use of only male mice in the MoA study submitted by the sponsor. The only other uncertainties were the lack of a time response relationship for gene expression of *Cyp2b10a* and for BROD microsomal enzyme activity in male mice at the 90-ppm dietary concentration.

I. Human Relevance

The following is the Registrant's conclusions on relevance of the MoA to humans:

Are key events in the animal MoA plausible in humans?

The relevance to a human of MoA should take into account available data from other chemicals which are associated with these key events (Cohen et al., 2004). Phenobarbital is the best studied chemical in laboratory animals and humans with respect to the events of tetraconazole-induced carcinogenicity. Phenobarbital is known to result in CAR-activation in mice and this, in turn, results in the induction of CYP2b isoforms (Holsapple et al., 2006). This process occurs in mice and appears to also occur in human hepatocytes (Yamada et al., 2009). Cell proliferation subsequent to phenobarbital dosing was not observed with human hepatocytes (Hirose, 2009), however, and this suggest that the key event of cell proliferation will not occur in the human liver. The absence of cell proliferation in human hepatocytes after phenobarbital administration is consistent with no finding of an increased incidence of carcinogenicity in human epidemiology studies of phenobarbital (IARC, 2001: Olsen et al., 1989). Based on the similarity of key events

in the MoAs of phenobarbital and tetraconazole and the absence of phenobarbital cell proliferation or carcinogenicity evidence in epidemiology studies the postulated MoA is not plausible in humans.

Meek et al, (2003) have suggested that a concordance table can be helpful in understanding the human relevance of rodent tumors to humans. The following concordance table presents the relevance conclusions regarding key events in tetraconazole-tumor induction in humans and mice:

Key event	Mice	Humans
Activation of CAR	Yes	Probably
Induction of CYP 2B	Yes	Probably
Increased cell proliferation	Yes	Not likely (based on analogy to phenobarbital)
Hepatocellular tumors	Yes	Not likely

Table taken from page 14 of the registrant's Human Relevancy Assessment (MRID 48930402)

Taking into account kinetic and dynamic factors, is the MoA plausible in humans?

The key events associated with the MoA occur at much higher dose levels that would be expected from human exposure to tetraconazole. Although CYP 2B is increased after four days of exposure at 90 ppm (14.4 mg/kg/day), the key event of cell proliferation is only seen at dietary concentration of 800 ppm (138.1 mg/kg bw/day) and greater in the mice. This dose level is also the dose level associated with an increased carcinogenic response in the mice and a clear increase in the number of animals with CAR-activation.

The US EPA estimates that dietary exposure to tetraconazole is no greater than 0.00014 mg/kg bw/day and occupational exposure is no greater than 0.00075 mg/kg bw/day (US EPA, 2005). The dose levels associated with key events are clearly much higher than the maximum anticipated exposures to humans. Based on the quantitative comparison of anticipated human exposure to tetraconazole to that of mice in the MoA and carcinogenicity studies the confidence in the conclusion that the MoA will not occur in humans is stronger than that based only on qualitative considerations.

Statement of confidence, analysis and implications

Taking into consideration all the above information it can be concluded that there is strong evidence for the postulated MoA for tetraconazole-induced hepatocellular tumors in male and female mice that requires CAR activation, CYP 2b induction and cellular proliferation. This MoA has been associated with other triazoles and phenobarbital. The data strongly support the conclusion that this MoA will not occur in humans due to the great difference in the expected human exposure and the dose levels in mice at which key events were observed in short-term and long-term dietary studies. In addition, phenobarbital, a well-studied pharmaceutical agent with

this MoA, has not been shown to induce liver tumors in humans chronically exposed to high dose levels.

CARC's Evaluation of Registrant's Human Relevance Argument

While the committee agrees there is sufficient evidence to establish a CAR/PXR-mediated MoA for liver tumors in mice, the data to support a lack of human relevance is less clear. The data do not exclude other potentially human relevant MoAs such as AhR and PPARα. In addition, no data were submitted to support a lack of mitogenic effect in humanized mice or human cells to support a lack of human relevance for the proposed MoA. The similarity of a select set of tetraconazole-mediated and phenobarbital-mediated responses in this MoA is insufficient to discount human relevance. The MoA is considered quantitatively plausible in humans.

V. COMMITTEE'S ASSESSMENT OF THE WEIGHT-OF-THE-EVIDENCE

Tetraconazole was carcinogenic to mice based on the following weight of evidence considerations: 1) There was a statistically significant ($p<0.01$) increase by pair-wise comparisons of the 800 ppm (118 and 140 mg/kg/day, for males and females, respectively) and 1250 ppm dose groups (217 and 224 mg/kg/day, for males and females, respectively) with the controls for hepatocellular adenomas, carcinomas (1250 ppm groups only) and combined adenomas/carcinomas in both sexes. The incidences of these tumors exceeded the range of historical controls. The dosing at 1250 ppm was considered by the Committee to be excessive due to increased mortality in both sexes. However, 800 ppm was considered to be adequate since mortality was comparable to controls and the tumor incidences were significantly increased in both sexes. In addition, there was a decrease in body weight gain, increased liver and kidney weights and the presence of non-neoplastic changes in various organs including bone, liver, lungs, kidney, testes, epididymides and ovaries. No evidence was found to support mutagenicity as a MoA for tetraconazole. Data supporting a non-genotoxic mitogenic MoA for liver tumors were presented to the committee. The CARC concluded that there is sufficient evidence with dose and time concordance to support the postulated MoA for liver cell tumors in male and female mice.

VI. CLASSIFICATION OF CARCINOGENIC POTENTIAL

The CARC considered the following factors in their weight-of-evidence deliberation on assessing the carcinogenic potential of Tetraconazole:

1. The Liver tumors observed in male and female mice were considered to be treatment related;

2. There were no treatment-related tumors in the rat;

3. The hypothesized MoA (activation of the CAR receptor leading to cell proliferation) was supported by studies that clearly indentified the sequence of key events, dose-response concordance and temporal relationship to tumor type. There was convincing evidence that the Liver cell carcinogenic effects are not likely to occur below a defined dose range.

The MoA data met the criteria established by the Agency;

4. There is no mutagenicity concern based on the results from the *in vitro* and *in vivo* genetic toxicity studies; and

5. Data are insufficient to conclude that the CAR-mediated MoA for rodent liver l carcinogenesis is not relevant to humans.

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC classified tetraconazole as "Not likely to be carcinogenic to humans at levels that do not cause increased cell proliferation in the liver." Quantification of carcinogenic potential is not required. The current RfD of 0.0073 mg/kg/day is based on a 1 year dog study in which nephrotoxicity (organ weight and histopathology) were seen at 2.95 mg/kg/day (LOAEL) and no adverse effects were observed at 0.73 mg/kg/day (NOAEL). This RfD would be protective of any liver effects caused by tetraconazole in the mouse carcinogenicity or mode of action studies at higher doses.

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